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Molecular Analysis of the *Azotobacter vinelandii glnA* Gene Encoding Glutamine Synthetase

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The gene encoding glutamine synthetase (GS), *glnA*, was cloned from *Azotobacter vinelandii* on a 6-kb *Eco*RI fragment that also carries the *ntrBC* genes. The DNA sequence of 1,952 bp including the GS-coding region was determined. An open reading frame of 467 amino acids indicated a gene product of M_r 51,747. Transcription of *glnA* occurred from a C residue located 32 bases upstream of an ATG considered to be the initiator codon because (i) it had a nearby potential ribosome-binding site and (ii) an open reading frame translated from this site indicated good N-terminal homology to 10 other procaryotic GSs. Sequences similar to the consensus RNA polymerase recognition sites at −10 and −35 were present at the appropriate distance upstream of the transcription initiation site. As expected from earlier genetic studies indicating that expression of *A. vinelandii glnA* did not depend on the *rpoN* (*ntrA*; σ^{54}) gene product, no σ^{54} recognition sequences were present, nor was there significant regulation of *glnA* expression by fixed nitrogen. Repeated attempts to construct glutamine auxotrophs by recombination of *glnA* insertion mutations were unsuccessful. Although the mutated DNA could be found by hybridization experiments in drug-resistant *A. vinelandii* transformants, the wild-type *glnA* region was always present. These results suggest that *glnA* mutations are lethal in *A. vinelandii*. In [^{14}C]glutamine uptake experiments, very little glutamine was incorporated into cells, suggesting that glutamine auxotrophs are nonviable because they cannot be supplied with sufficient glutamine to support growth.

Glutamine plays a pivotal role in nitrogen metabolism in bacteria, serving as an amino group donor or as a substrate in biosynthetic reactions. The synthesis of glutamine from glutamate and ammonia is catalyzed by the enzyme glutamine synthetase (GS; EC 6.3.1.2). In the nitrogen-fixing *Azotobacter vinelandii*, GS is thought to be solely responsible for the assimilation of ammonia; glutamate dehydrogenase, while present in many other bacteria, is probably absent from *A. vinelandii* (for a review, see reference 23).

The gene encoding glutamine synthetase, *glnA*, was cloned from *A. vinelandii* and shown to be linked to *ntrC*, a gene involved in the regulation of nitrogen metabolism (52). A region between *glnA* and *ntrC* hybridized to the *ntrB* gene from *Klebsiella pneumoniae* (our unpublished results). The organization of the *glnA-ntrBC* region in *A. vinelandii* is therefore similar to that in enteric bacteria, in which *glnA* is upstream of the *ntrB* (*glnL*) and *ntrC* (*glnG*) genes in an operon with complex regulation (for reviews, see references 28, 32, and 40). In *Klebsiella pneumoniae* (and other enteric organisms), expression of *glnA* can occur from two different promoters; one of these, $glnAp_2$, requires sigma factor σ^{54}, encoded by *rpoN* (*ntrA*), for recognition and shows the characteristic −12 and −24 consensus sequences indicative of σ^{54}-dependent promoters. Expression from this promoter also requires the *ntrC* gene product (also called NR1 [40]), which binds to sequences located further upstream and activates transcription of the *glnA-ntrBC* operon in conditions of low availability of fixed nitrogen. Expression from the other promoter, $glnAp_1$, located upstream of the σ^{54} recognition sequence, is more typical of *Escherichia coli* promoters characterized by the consensus sequences present at −10 and −35 in relation to the transcription start site, which are recognized by the abundant form of RNA polymerase containing the sigma factor σ^{70}. Expression of the *ntrBC* genes, downstream of *glnA*, can occur in enteric organisms from either $glnAp_1$, $glnAp_2$, or a σ^{70}-dependent promoter, p_3, adjacent to *ntrB*, downstream of which is a site where NtrC binds and represses *ntrBC* expression in cells growing with high levels of fixed nitrogen.

In contrast to enteric organisms, genetic evidence indicated that expression of *glnA* and the downstream *ntrC* gene in *A. vinelandii* occurs from separate promoters, neither of which requires RpoN or NtrC for expression (43, 52). We report here studies of the *A. vinelandii glnA* gene which include its DNA sequence, its ability to be highly expressed in *E. coli*, the size and start point of the *glnA* transcript, and expression experiments indicating that *glnA* is not subject to control by levels of fixed nitrogen in *A. vinelandii*. An attempt was made to construct glutamine auxotrophs of *A. vinelandii* by introducing *glnA* Tn*5* or Km^r cartridge insertion mutations into the genome. The mutated DNA failed to become stably integrated even after a long period of repeated subculture on kanamycin, indicating that *glnA* mutations are probably lethal in *A. vinelandii*. This characteristic may be due to an inability of the organism to accumulate glutamine, as suggested by [^{14}C]glutamine incorporation experiments.

MATERIALS AND METHODS

Bacterial strains and growth conditions. *A. vinelandii* UW136 (3) was the wild-type organism used in this study. Cultures were grown at 30°C on Burk nitrogen-free medium

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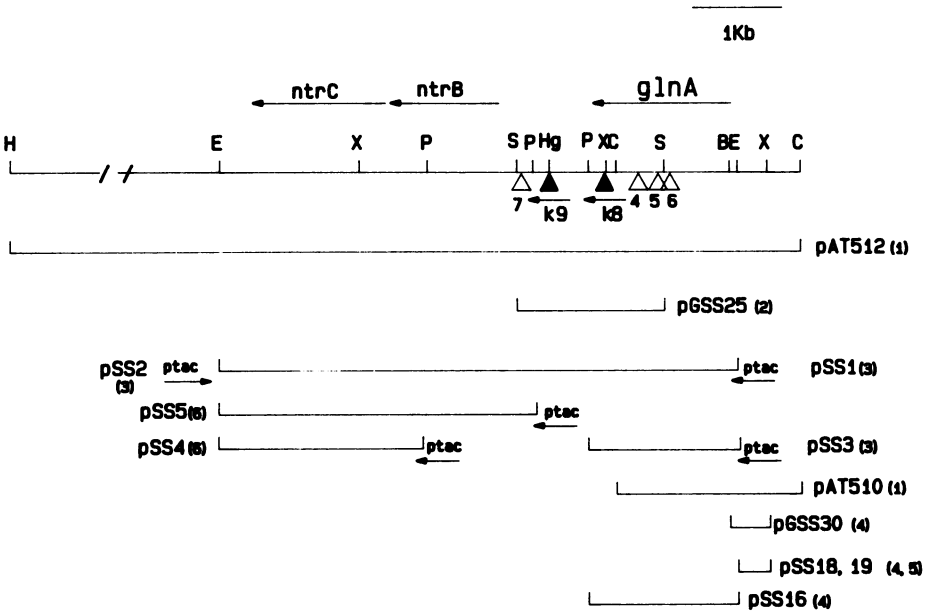

FIG. 1. The *glnA-ntrBC* region of *A. vinelandii*. The extents of the *glnA* region sequenced in this work and of the *ntrBC* sequenced regions (unpublished results) are indicated by arrows. Subclones of this region are indicated by plasmid names followed by cloning vectors (1, pBR325; 2, pSUP201-1; 3, pKK223-3; 4, pTZ18; 5, pTZ19; 6, pMM40). Other plasmids carrying the 6-kb *glnA-ntrBC Eco*RI fragment were subcloned from pAT523 (52). They include pGSS20, which was used in the mutagenesis studies, and pNFL1 and pNFL2, used for sequencing (see Table 1). Open triangles and numbers indicate sites of Tn*5* inserts in pLV50 (4 and 6) and Tn*5*-B21 inserts in pGSS23 and pGSS24 (5 and 7) (for plasmids in which Tn*5* or Tn*5*-B21 was inserted, see Table 1). Closed triangles and numbers preceded by k indicate sites of insertion of the KIXX cartridge in pGSS22 and pGSS27 (see Table 1). See reference 52 for original construction of pAT512 and pAT510. Restriction sites: H, *Hin*dIII; E, *Eco*RI; X, *Xho*I; P, *Pst*I; S, *Sph*I; Hg, *Hgi*EII; C, *Cla*I; B, *Bam*HI.

(38) containing 2% sucrose as a carbon source with 15 mM ammonium acetate (BSN), with 10 mM sodium nitrate, with 10 mM urea, or with no added source of fixed nitrogen (BS).

The *E. coli* strain used was the *glnA ntrBC* deletion mutant ET8894 (30). To determine the ability of various plasmids to complement ET8894 for its glutamine auxotrophy, growth was tested on minimal medium as described previously (52), with the addition of 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) when expression was from the vector-encoded *tac* promoter.

Antibiotic concentrations (in micrograms per milliliter) used for the selection of transformants in *E. coli* and *A. vinelandii*, respectively, were as follows: kanamycin, 35 and 2; tetracycline, 10 and 5; carbenicillin, 50 and 10; and chloramphenicol, 35 and 50.

Plasmids. The plasmids used are described in Fig. 1 or Table 1. Standard methods were used for their construction and for transformation into *E. coli* (31).

Enzyme assays. GS was assayed by using the γ-glutamyltransferase assay in the presence of 0.3 mM Mn^{2+} and 60 mM $MgCl_2$ (45). β-Galactosidase was assayed as described by Miller (33); results are averages from duplicate samples from two or more experiments.

Identification of gene products. Plasmids pSS1 to pSS5 were transferred into *E. coli* ET8894. Overnight cultures grown on LB containing 10 mM ammonium chloride and 200 mg of glutamine ml^{-1} were diluted in the same medium to a concentration of 2×10^7 to 5×10^7 cells ml^{-1} and incubated for 2 h at 30°C. Each culture was then divided into two samples, and IPTG was added to one at a final concentration of 1 mM. Both samples were incubated for an additional 4 h before being harvested. Cells were lysed by incubation in protein sample buffer for 3 min in boiling water, after which samples were run on 10% acrylamide Laemmli gels (29). Molecular weight markers were MW-SDS-200 (Sigma).

Sequencing methods. The entire 6-kb *Eco*RI fragment carrying the *glnA* and *ntrBC* genes in pAT523 (52) and smaller regions with only *glnA* were subcloned from pAT523 into pUBS1, yielding pNFL1 and pNFL2, respectively, or from pAT510 into pTZ18 and pTZ19, yielding pSS18 and pSS19, respectively (Fig. 1; Table 1). Digestion of these plasmids with exonuclease III generated a set of deletions spanning *glnA* that were appropriate for sequencing both strands of DNA. The dideoxynucleotide chain termination sequencing method was used with a denatured plasmid DNA template (36).

The sequence analysis software package of the University of Wisconsin Genetics Computer Group (11) was used to compile and analyze DNA and protein sequences.

Analysis of transcripts. *A. vinelandii* UW136 cultures were grown at 30°C with reciprocal shaking in Burk sucrose medium with different nitrogen sources as indicated above. Samples (10 ml) of cells were added to 1 ml of RNA lysis solution (0.5 M Tris hydrochloride [pH 8.0], 0.2 M EDTA, 10% [wt/vol] sodium dodecyl sulfate [SDS]) with gentle mixing. An equal volume of phenol–*m*-cresol–8-hydroxyquinoline (1,000:140:0.4) was added, and the contents were gently mixed. After 5 min on ice, the tubes were centrifuged at room temperature at 1,500 *g* for 5 min. An 8-ml sample of the aqueous phase was removed; nucleic acids were precipitated with ethanol. After centrifugation, the pellet was dissolved in 50 μl of H_2O and electrophoresed on formaldehyde-agarose gels. Electrophoresis and Northern (RNA) blots of gels onto Hybond-N were performed as described by the manufacturer (Amersham International plc). Blots were

TABLE 1. Plasmids and transposons used[a]

Plasmid or transposon	Description	Reference or source
Plasmids		
pAT512.Km	6-kb *Eco*RI fragment of pAT512 replaced with 1.4-kb Km^r cartridge from pUC4K	52; this report
pAT523	6-kb *Eco*RI *glnA-ntrBC* fragment cloned in pBR325	52
pF7*lac*	Tn*7* inserted in pF7 for selection of *lacI*^{q}	This report
pGSS20	6-kb *Eco*RI *glnA-ntrBC* fragment cloned in pSUP201-1	This report
pGSS22	KIXX (Km^r) cartridge inserted in *Xho*I site in *glnA* in pGSS20 (Fig. 1, insert K8)	This report
pGSS23	Tn*5*-B21 inserted in *glnA* in pGSS20 (Fig. 1, insert 5)	49; this report
pGSS24	Tn*5*-B21 inserted in *glnA-ntrB* intergenic region in pGSS20 (Fig. 1, insert 7)	This report
pGSS27	KIXX (Km^r) cartridge inserted in *Hgi*EII site in *glnA-ntrB* intergenic region of pGSS25 (Fig. 1, insert K9)	This report
pKK223-3	Vector for expression from *tac* promoter	Pharmacia P-L Biochemicals plc
pLV50	pLAFR1 cosmid isolated from *A. vinelandii* genomic library with 28-kb insert including the *glnA-ntrBC* region	52
pLV50::Tn5-6	pLV50 with Tn*5* in *glnA* (Fig. 1, insert 6)	This report
pLV50::Tn5-4	pLV50 with Tn*5* in *glnA* (Fig. 1, insert 4)	This report
pNFL1, pNFL2	6-kb *Eco*RI *glnA-ntrBC* fragment cloned in two orientations in pUBS1	This report
pSUP201-1	Mob$^+$ derivation of pBR325	48
pTZ18R, pTZ19R	Multifunctional cloning and sequencing vectors	Pharmacia P-L Biochemicals plc
pUBS1	Polylinker from Bluescript (Stratagene) cloned into pUC7	36
pUC4K	Source of Km^r (Tn*903*) cartridge	54
pUC4-KIXX	Source of Km^r (Tn*5*) cartridge	Pharmacia P-L Biochemicals plc
pMM40	pKK223-3 with *lacI*^{q}	24
Transposon		
Tn*5*-B21	Tn*5* derivative with Tc^r and *lacZ* carried on λ *b*221 *c*I857 *P*am80	49

[a] In addition to those shown in Fig. 1.

hybridized to a ^{32}P-labeled *glnA* fragment isolated from pSS16.

Primer extension experiments. The oligonucleotide used for priming reverse transcriptase synthesis of DNA from *glnA* mRNA, prepared as described above, had the following sequence: 5′-TTGATCAGTTGAAGCGACT-3′ (see Fig. 4a). A 2-pmol amount of oligonucleotide was labeled at the 5′ end with [^{32}P]dATP, using T4 polynucleotide kinase (31). After annealing to 10 μg of RNA, the primer was used in a reaction with reverse transcriptase and deoxynucleoside triphosphates. The extended oligonucleotide was sized on a 7% polyacrylamide–8 M urea sequencing gel. The size of the product was determined by comparison with a sequencing ladder obtained with dideoxynucleotide reactions in which 1 pmol of the same oligonucleotide was used with single-stranded template prepared from pGSS30.

Construction and characterization of *A. vinelandii* mutants. Preparation of competent *A. vinelandii* UW136 and transformation with either chromosomal or plasmid DNA have been described previously (52). In the attempts to isolate *glnA*::Tn*5*, *glnA*-KIXX, or *glnA ntrBC* deletion-Km^r cartridge mutants, competent cells were transformed with pLV50::Tn5-6, pLV50::Tn5-4, pGSS23, pGSS22, or pAT512.Km (Table 1) and plated on BSN plus kanamycin (or tetracycline for pGSS23) supplemented with glutamine at either 1 or 2 mg ml^{-1} plus sometimes glutamate at 0.5 μg ml^{-1}. To test whether transformants had recombined the entire plasmid into the genome by a single-crossover event, transformants were tested on BSN supplemented with glutamine and tetracycline or chloramphenicol, depending on the vector used. Transformants that were resistant only to kanamycin (or tetracycline for pGSS23) were tested further (see Results).

DNA hybridization studies. Restriction fragments of genomic DNA, prepared as previously described (41), were separated by electrophoresis on agarose gels and transferred to Hybond-N membranes (Amersham) by capillary Southern blotting. Probes were prepared by running digested plasmid DNA on 0.7% low-melting-point agarose gels, using TAE buffer. The desired fragment was excised, and water was added at a ratio of 1.5 μl per mg of gel fragment. After incubation for 10 min in a boiling water bath, the DNA solution was labeled with [^{32}P]dCTP by the random pentanucleotide priming method (12). Hybridization to blots was performed according to Hybond-N protocols supplied by Amersham. Dried blots were autoradiographed on Kodak X-Omat R X-ray film.

DNAs used as probes were as follows: for Tn*5*, the 2.45-kb *Xho*I doublet from pBR325::Tn5; and for *A. vinelandii glnA*, the 6-kb *Eco*RI insert of pAT523 or the 1.2-kb *Eco*RI-*Cla*I fragment from pAT520.

Glutamine uptake assays. Cells from 10 ml of a mid-log-phase culture of *A. vinelandii* UW136 grown on BSN or BS were washed once and then resuspended in the same volume of medium in a 100-ml flask. This culture was incubated with vigorous shaking in a 30°C water bath for 1 h before the assays were performed. For each assay, a glass vial containing uniformly labeled [^{14}C]glutamine (to give a final concentration of 3.75 μM when the cells were added; specific activity, 285 mCi mmol^{-1}; Amersham) was incubated at 30°C for 1 min. Assays were started by the addition of 280 μl of the culture. Samples of 40 μl were removed at various time intervals and immediately filtered onto a polycarbonate membrane (Nuclepore) which rested on a prewetted piece of Whatman no. 1 filter paper on a filtering apparatus. After a sample had been filtered (approximately 5 s) and dried briefly, the polycarbonate membrane was transferred to a scintillation vial containing 5 ml of Cocktail T (BDH), and radioactivity was measured.

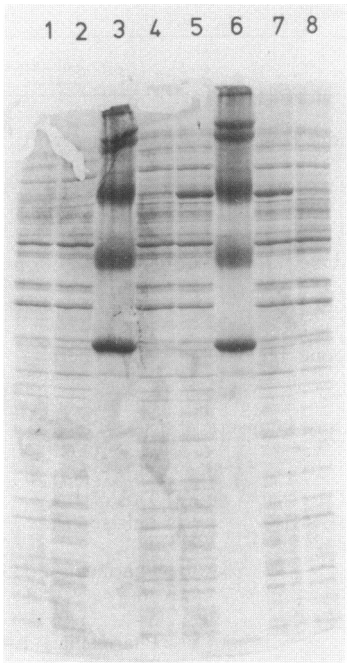

FIG. 2. Coomassie-stained gels after SDS-polyacrylamide gel electrophoresis of extracts made from *E. coli* ET8894 carrying plasmids of the *glnA-ntrBC* region shown in Fig. 1. The plasmids and growth conditions used were as follows: lane 1, pSS4, with IPTG; lane 2, pSS5, with IPTG; lane 4, pSS1 and pF7*lac*, no IPTG; lane 5, pSS1 and pF7*lac*, with IPTG; lane 7, pSS3 and pF7*lac*, with IPTG; and lane 8, pSS3 and pF7*lac*, no IPTG. Markers of M_r 205,000, 116,000, 97,400, 66,000, 45,000, and 29,000 were run in lanes 3 and 6.

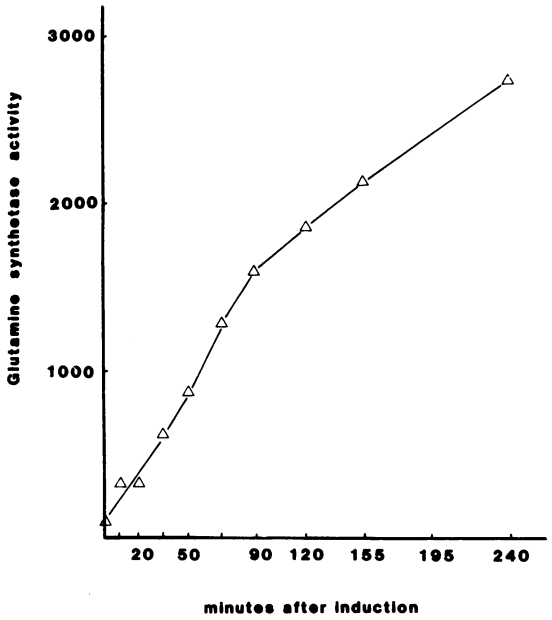

FIG. 3. GS activity of ET8894(pF7*lac*)(pSS1) after addition of IPTG. Activity was measured as the amount of γ-hydroxamate produced in the transferase assay of GS in the presence of 0.3 mM $MnCl_2$ and 60 mM $MgCl_2$ (45). No activity was detected in ET8894(pF7*lac*) or in ET8894(pF7*lac*)(pSS2) (see Fig. 1).

RESULTS

Proteins expressed from the *A. vinelandii glnA-ntrBC* region in *E. coli*. The 6-kb *Eco*RI fragment containing the *A. vinelandii glnA-ntrBC* region was cloned into pKK223-3, yielding pSS1, such that expression of the *A. vinelandii* genes was directed from the vector-encoded *tac* promoter (Fig. 1). Earlier results indicated that the *glnA* promoter was not included on the 6-kb *Eco*RI fragment (52). However, the *glnA* coding region was intact, since *glnA* mutants of *E. coli* or *K. pneumoniae* could be complemented when the fragment was cloned such that the vector provided a promoter for expression. pSS1 directed the synthesis of a polypeptide of M_r approximately 62,000 after the addition of IPTG (Fig. 2). The appearance of this polypeptide corresponded to the IPTG-inducible appearance of GS γ-glutamyltransferase activity in ET8894 carrying pSS1 and the *lacI*^{q} gene on pF7*lac* (Fig. 3). The presence of *lacI*^{q} in the strain was necessary to prevent uninduced expression from the *tac* promoter (data not shown). Neither this polypeptide nor GS activity was present in IPTG-induced ET8894(pF7*lac*) carrying pSS2, a construct in which the 6-kb *Eco*RI fragment was in the reverse orientation (data not shown). The polypeptide was present in cultures carrying pSS3, a plasmid which could complement the glutamine auxotrophy in ET8894 and which was derived from pSS1 by deleting 4.4 kb of *A. vinelandii* DNA carrying the *ntrBC* region. This polypeptide can therefore only be the *glnA* gene product of *A. vinelandii*, although its size is larger by 5.5 to 9 kDa than the sizes reported previously for the subunit of purified GS from this organism (25, 47). No additional proteins corresponding to the molecular weights of the *ntrB* or *ntrC* gene product, expected to be 36,000 or 56,000, respectively, from DNA sequencing results (unpublished results), were detected after IPTG induction of pSS1 in ET8894.

Sequence of the *glnA* gene and comparison of the translated amino acid sequence with sequences of GS enzymes from other organisms. The nucleotide sequence of 1,952 bp between the *Xho*I and *Pst*I sites of the *glnA* region shown in Fig. 1 was determined by dideoxynucleotide sequencing of pNFL1 and pNFL2 (and their deletion derivatives), pSS18, and pSS19 (Fig. 1 and Materials and Methods). Both strands of this region were sequenced. The sequence (Fig. 4) contains an open reading frame from the ATG codon at position 478 to the TGA stop codon at 1879 (1,401 bp) which encodes a polypeptide of 467 amino acids with a molecular weight of 51,747. The codon usage of this open reading frame conforms well to that averaged for *A. vinelandii* nitrogen fixation genes (Fig. 4b); its G+C content is 62% (the *A. vinelandii* genome has 66% G+C; 20). The probable ribosome-binding site, GGAGG, similar to the consensus sequence complementary to 16S rRNA in procaryotes (46), is 10 bp upstream of the ATG codon at position 478. A G+C-rich region of 24 bp showing significant dyad symmetry followed by a string of six T residues was located beginning at position 1900, 19 bp after the translational stop codon; this is a likely site for termination of transcription. The mRNA hairpin loop predicted from this sequence has a ΔG of −18.2 kcal (ca. −76.1 kJ)/mol (52).

A comparison of the *A. vinelandii glnA* amino acid content derived from its DNA sequence with the results of amino acid analysis of purified GS from *A. vinelandii* (25) showed a good correspondence (Table 2). An abundance of acidic residues indicates by computer calculation a pI of 4.97 for the protein. A comparison of the deduced amino acid sequence with 10 other procaryotic GS sequences is shown in

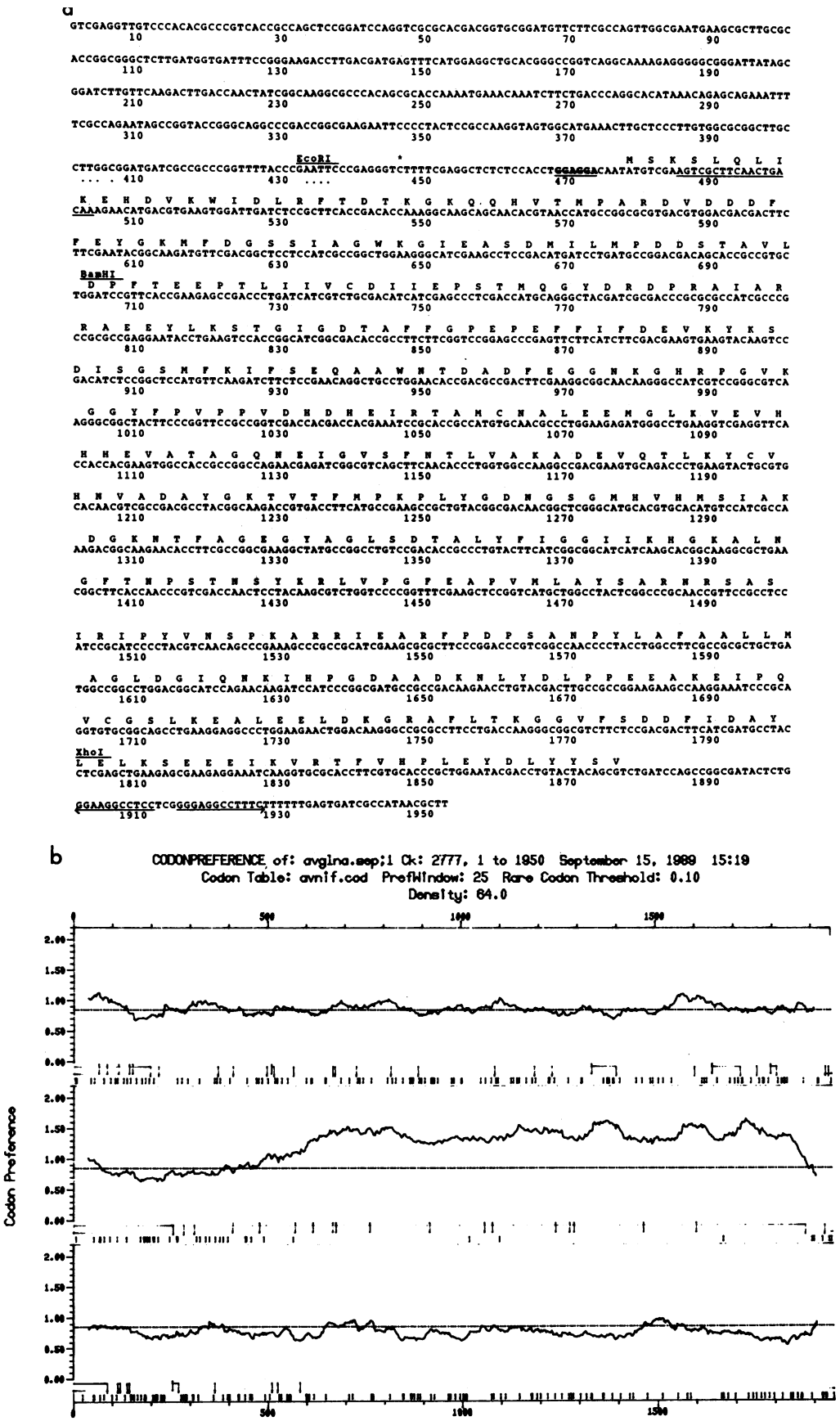

FIG. 4. (a) Nucleotide sequence of the *glnA* gene of *A. vinelandii* and 480 bp upstream. The sequence was obtained for both strands of DNA by the dideoxynucleotide chain termination method. Features indicated include putative −10 and −35 RNA polymerase recognition sequences (. . .), transcription start site (*), ribosome-binding site (underlined), and particular restriction sites (*Eco*RI, *Bam*HI, and *Xho*I). Also underlined is the sequence of the oligonucleotide used in primer extension experiments to identify the *glnA* transcription start site (see Fig. 7) and the terminator sequence located at the end of the gene. (b) Codon preference analysis of the three translation frames of the *glnA* region. The codon preference table used for comparison was compiled from published sequences of nitrogen fixation genes in *A. vinelandii*.

TABLE 2. Amino acid residues per subunit of *A. vinelandii* GS protein

Amino acid	No./subunit	
	From sequence	From analysis of purified protein[a]
Alanine	41	44
Cysteine	4	4
Aspartic acid	37	56[b]
Asparagine	16	
Glutamic acid	34	46[c]
Glutamine	9	
Phenylalanine	26	26
Glycine	39	41
Histidine	14	14
Isoleucine	28	25
Lysine	32	32
Leucine	31	32
Methionine	14	13
Proline	27	28
Arginine	17	17
Serine	27	27
Threonine	22	22
Valine	27	28
Tryptophan	3	3
Tyrosine	19	20
Total	467	478

[a] From reference 25.
[b] Total for aspartic acid plus asparagine.
[c] Total for glutamic acid plus glutamine.

Fig. 5. The alignment and consensus sequence was obtained by using the GAP and PRETTY programs of the University of Wisconsin Genetics Computer Group software package (11). The conserved tyrosine residue at amino acid position 407 in the *A. vinelandii* GS protein is probably the site of adenylylation, since it corresponds well to that found in *E. coli* and other organisms (see reference 44 and references in the legend to Fig. 5); also, *A. vinelandii* GS is known to be adenylylated in vivo in response to addition of ammonium (25, 26, 47). Other conserved regions, corresponding to those predicted by Almassy et al. (1) to be involved in the formation of the active site, are underlined in Fig. 5. The degree of homology between *A. vinelandii* GS and those of other procaryotes is from 70 to 72% identical residues (*Salmonella typhimurium*, *E. coli*, and *Methylococcus capsulatus*) to 43 to 44% (*Clostridium acetobutylicum* and *Bacillus subtilis*) (Fig. 5).

Analysis of transcripts and initiation site. It was of interest to determine the size of mRNA transcripts in *A. vinelandii* to see whether any downstream sequences might be cotranscribed with *glnA* as occurs in enteric organisms. Northern blots of RNA prepared from strain UW136 grown in BS, BSN, BS plus nitrate, and BS plus urea (see Materials and Methods) were hybridized to a *glnA*-specific probe labeled with [^{32}P]dCTP. In all four cases, a single RNA species of approximately 1.5 kb hybridized to the probe (Fig. 6), indicating that the *glnA* gene is probably a single transcriptional unit. The variable intensity of hybridization observed for the four growth conditions does not necessarily indicate that *glnA* expression is regulated by fixed N supply, since the amount of RNA loaded in each lane was not determined. A *glnA-lacZ* transcriptional fusion in strain MV610, constructed by transposition of Tn*5*-B21*lacZ* (49) into the *glnA* gene in pGSS23 followed by transformation into the genome of *A. vinelandii* UW136, expressed β-galactosidase similarly after growth on different nitrogen sources. Although the *glnA*::Tn*5*-B21 transformants were unstable after subculturing on tetracycline-free medium (see below), β-galactosidase activities ranged from 612 to 884 Miller units (33) in cultures supplied with tetracycline and N_2, nitrate, ammonium, or urea.

Primer extension experiments identified the transcription initiation site for the *glnA* gene. Using an oligonucleotide primer for DNA synthesis by reverse transcriptase from position 503, it was shown that mRNA transcripts prepared from cells grown in 15 mM ammonium acetate began at the C residue at position 446 (Fig. 7), 32 bp upstream of the translation initiation codon (Fig. 5). Sequences approximating the procaryotic −10 and −35 consensus for recognition by σ^{70} (15) were found at the appropriate locations upstream of the transcription start site (* in Fig. 4a).

Attempts to construct a *glnA* mutant in *A. vinelandii*. The construction of three types of *A. vinelandii glnA* mutants was attempted. The first was a mutant with Tn*5* inserted in *glnA*; the second had the KIXX Km^r cartridge inserted at the *Xho*I site in *glnA*; the third was one in which the 6-kb *Eco*RI fragment encoding *glnA* and *ntrBC* was deleted with replacement by another Km^r cartridge (see Materials and Methods). The plasmids carrying these mutations are pLV50::Tn*5*-6 (or pLV50::Tn*5*-4), pGSS22, and pAT512.Km (Fig. 1; Table 1). Wild-type *A. vinelandii* was transformed with plasmid DNA and subsequently plated on medium containing kanamycin plus glutamine. However, no glutamine auxotrophs were found among the transformants. This result was surprising because this method of mutagenesis of *A. vinelandii* had previously yielded many *nif* and *ntr* mutants (e.g., references 16 and 52). To test the possibility that *A. vinelandii* has two GS genes, as do *Rhizobium* (7, 10, 13) and *Agrobacterium* (14) species, extracts from wild-type cells and from two Km^r transformants grown on either ammonium or urea were prepared. Samples from the extracts were run on nondenaturing gels and stained for GS activity under conditions that resolve both GS enzymes from *Rhizobium leguminosarum* (13). Only a single band was observed in all cases (data not shown). This finding suggested that *A. vinelandii* contains only one type of GS. Therefore, the events occurring in *A. vinelandii* after introduction of DNA carrying *glnA*::Tn*5* were examined.

When pLV50::Tn*5*-6 DNA was used to transform *A. vinelandii*, Km^r isolates were obtained on BSN plus kanamycin supplemented with glutamine at 1 to 2 mg ml^{-1}, a concentration more than sufficient to allow growth of glutamine auxotrophs of *E. coli* and other enteric organisms on minimal medium. These isolates were sensitive to tetracycline, the drug resistance encoded by pLV50, which suggested that the mutated gene had recombined into the chromosome by a double-crossover event. Although pLV50 and derivatives can replicate in *A. vinelandii*, few Tc^r transformants were obtained. It is possible that large plasmids are often linearized by nuclease activity during the transformation process. After being restreaked several times to allow for chromosome segregation, several Km^r colonies were examined, and all were capable of growth on medium without glutamine. Resistance to kanamycin was unstable in about half of the isolates; i.e., they became sensitive to kanamycin after a single restreak on medium without kanamycin.

To determine whether the proposed gene replacement had occurred and to investigate the basis for the instability of kanamycin resistance, the experiment was repeated, and at each serial subculture of a transformant, the stability of kanamycin resistance was determined. In addition, DNA

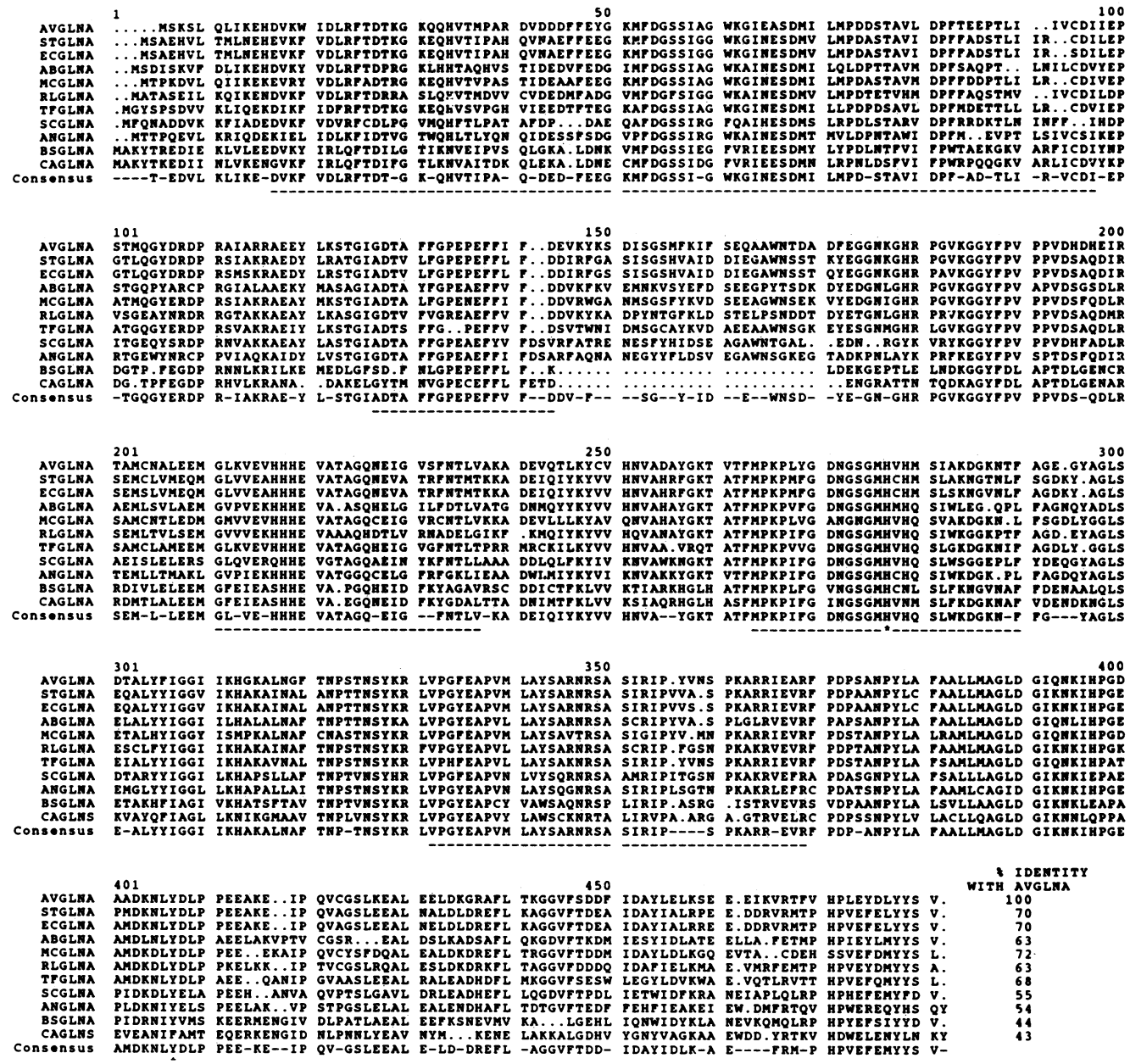

FIG. 5. Alignment of amino acid sequences of GS proteins from *A. vinelandii* and 10 other procaryotes. The underlined regions are those considered particularly important for enzyme function, with the histidine residue at 277 at the active site, as deduced from X-ray crystallography of *S. typhimurium* GS (1). The conserved tyrosine at 407 is adenylylated in most organisms in response to ammonium addition. Percentage of identical residues between *A. vinelandii* GS (AUGLNA) and the others is indicated. The alignment and consensus shown were the output of sequence analysis, using the programs GAP and PRETTY of the University of Wisconsin Genetics Computer Group software package (11). The weighting parameters for PRETTY were plurality = 5 (a consensus was assigned only if 5 of the 11 residues were identical or conservative substitutions) and threshold = 1 (all sequences counted equally). Symbols for the other organisms compared and references for their sequences are as follows: ST, *S. typhimurium* (17); EC, *E. coli* (34); AB, *Azospirillum brasilense* (4); MC, *M. capsulatus* (6); RL, *R. leguminosarum* (8); TF, *Thiobacillus ferrooxidans* (39); SC, *Streptomyces coelicolor* (56); AN, *Anabaena* sp. (53); BS, *B. subtilis* (50); and CA, *C. acetobutylicum* (18).

was isolated from certain subcultures and, after digestion with restriction enzymes and Southern blotting, hybridized to *glnA* or Tn*5* probes. Two isolates from the transformation with pLV50::Tn5-4 (strains MV504a and MV504b) and two from the transformation with pLV50::Tn5-6 (MV506a and MV506b) were studied. Of these four transformants, kanamycin resistance was unstable in MV504a and in MV506a initially and remained so through 10 serial subcultures. The resistance to kanamycin in strains MV504b and MV506b was always stable. All four isolates were sensitive to tetracycline throughout subculturing.

None of the transformants ever required glutamine for growth. Hybridization experiments showed that all four transformants possessed wild-type and mutant copies of the *glnA* gene. This is possible since *A. vinelandii* has been shown to have up to 40 chromosome equivalents of DNA per

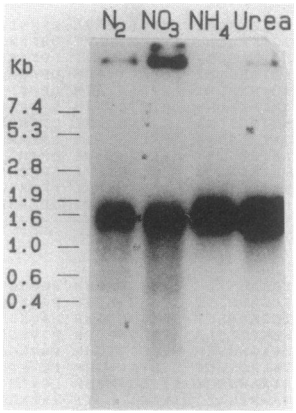

FIG. 6. Sizes of transcripts hybridizing to a *glnA* probe from cultures of *A. vinelandii* grown on different N sources. RNA preparation, electrophoresis, Northern blotting, and hybridization protocols are given in Materials and Methods. Sizes are shown in relation to the separation of RNA markers (purchased from Boehringer) run on the same gel.

cell (42). The results obtained when *Eco*RI-*Hin*dIII-digested DNA was probed with the 6-kb *Eco*RI *glnA-ntrBC* fragment are shown in Fig. 8. Though the intensities of the hybridizing bands varied, indicating a change in the relative proportion of wild-type to mutant gene copies, both types were present even in subculture 10 of MV504a and MV506a (not shown). Derivatives of MV504a and MV506a sensitive to kanamycin were isolated after subculture 9. In these two strains, only the wild-type *glnA* gene was present (data not shown). This finding indicated that the mutated copy of *glnA* was rapidly lost when selective pressure for kanamycin resistance was removed.

It was also not possible to construct a mutant in which the entire *glnA-ntrBC* region was deleted. When plasmid pAT512.Km DNA was used to transform *A. vinelandii*, all Km^r transfsormants tested were also Ap^r and Cm^r, the two plasmid-encoded drug resistances. This finding suggested

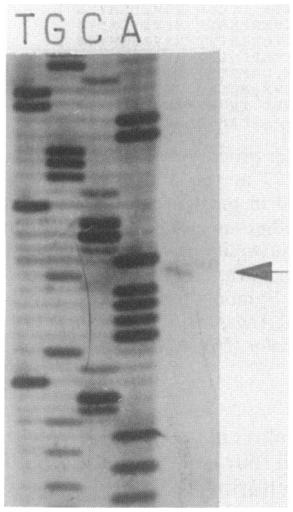

FIG. 7. Primer extension into the *glnA* promoter region to identify the transcription initiation site. The product of extension of the primer oligonucleotide shown in Fig. 4 by reverse transcriptase after its hybridization to RNA was run on a sequencing gel is shown (←). The DNA sequence of the same region on pGSS30 is also shown.

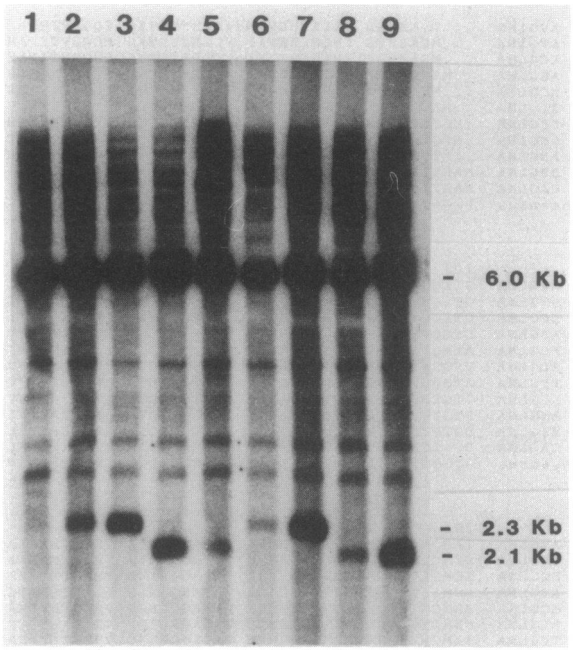

FIG. 8. Molecular characterization of presumptive *glnA*::Tn*5* mutants. DNA was isolated from the wild type (lane 1) or from mutant strain MV504a (lane 2), MV504b (lane 3), MV506a (lane 4), or MV506b (lane 5) after the second subculture or from MV504a (lane 6), MV504b (lane 7), MV506a (lane 8), or MV506b (lane 9) after the third subculture. DNA was digested with *Eco*RI and *Hin*dIII, Southern blotted, and then hybridized to the 6-kb *Eco*RI *glnA-ntrBC* fragment. Since there are no *Hin*dIII sites in the 6-kb fragment and two sites in Tn*5*, the wild-type *glnA* region is indicated by a 6-kb hybridizing fragment, whereas a copy of *glnA*::Tn*5-4* is indicated by 6.1- and 2.3-kb fragments and a copy of *glnA*::Tn*5-6* is indicated by 6.3- and 2.1-kb fragments.

that the entire plasmid was being integrated into the chromosome. To prevent this, pAT512.Km was first linearized with *Sca*I and then used to transform *A. vinelandii*. In this instance, all Km^r transformants were Ap^s and Cm^s. However, in all transformants tested, the resistance to kanamycin was lost if selective pressure was not maintained. These transformants did not require glutamine for growth, and hybridization of their DNAs with the *glnA ntrBC* probe indicated that only the wild-type *glnA* region was present.

The third type of *glnA* mutant sought was one in which the KIXX cartridge was inserted at the *Xho*I site in the 3′ end of *glnA*; the orientation of insertion in the resulting plasmid, pGSS22, would have allowed expression of any downstream regions. As before, none of the Km^r transformants required glutamine for growth; after subculturing a few times on medium without kanamycin, they became Km^s.

Uptake of [^{14}C]glutamine and [^{14}C]glutamate. A possible explanation for the apparent lethality of *glnA* mutations in *A. vinelandii* is that glutamine is poorly transported, and therefore glutamine auxotrophs cannot be provided with an essential nutrient. Therefore, the uptake of glutamine, and of glutamate for comparison, was measured in strain UW136 grown on BSN to which uniformly labeled [^{14}C]glutamine or [^{14}C]glutamate was added (see Materials and Methods). The rate of glutamine uptake was 0.037 to 0.049 nmol min^{-1} mg of $protein^{-1}$. A similar rate was obtained for cells grown on N_2, ammonium (15 mM), or urea (10 mM) as the nitrogen source. The rate of glutamate uptake was about 0.06 nmol min^{-1} mg of $protein^{-1}$. However, during the 10-min time

course of the assay, a significant amount of total ^{14}C label was lost as CO_2 in cells from all cultures examined. Loss of label was not detected before about 2 min, but by 5 min 90% of the ^{14}C added as glutamate and 40% of that added as glutamine was absorbed onto filter paper saturated with 30% KOH present in a side-arm tube.

DISCUSSION

The subunits of *A. vinelandii* GS enzyme determined from the *glnA* sequence have an M_r of 51,747. This value compares well with that of 53,000 or 56,500 for the subunits found in purified *A. vinelandii* GS (25, 47), as does the amino acid content deduced from the sequence and compared with the analysis of purified protein (25). In contrast, the subunit molecular weight observed on SDS-polyacrylamide gels of the protein expressed after induction by IPTG of *glnA* expressed from the *tac* promoter in *E. coli* was estimated to be 60,000 to 62,000. The appearance of this protein band correlated directly with an increase in GS activity measured in extracts. A large fusion protein between GS and vector-encoded polypeptide is ruled out because there is no significant distance between the start of *glnA* and the *tac* promoter. The *A. vinelandii* GS protein is fairly acidic, with a pI of 4.97; acidic proteins such as nitrogenase Mo-Fe protein subunits are known to migrate anomalously slowly on SDS-gels (21). GS from *M. capsulatus* also ran on SDS-gels as an M_r-60,000 protein, whereas its translated DNA sequence indicated an M_r-51,717 gene product (6). In contrast, however, the equally acidic GS proteins of *Azospirillum brasilense* and *Streptomyces coelicolor* expressed in *E. coli* migrate on SDS-gels to a position corresponding to their true molecular weight, about M_r 50,000 (4, 5, 56). The reason for the difference between migration on SDS-gels of *A. vinelandii* and *M. capsulatus* GS subunits and the others is not known but could be due to differences in the purity of the SDS used in the different laboratories, an effect demonstrated by Kennedy et al. (21) for nitrogenase proteins.

The transcription start site of the *glnA* gene was at a C residue located 32 bp upstream of the ATG start codon. Sequences upstream of the transcription start site show some conformity with the consensus −10 and −35 regions of procaryotic promoters: at −10, CCGAAT has three matches with the consensus TATAAT, while at −35, TTGGCG matches four bases with the consensus TTGACA (see Fig. 4a). A compilation of known −10 and −35 sequences of *E. coli* promoters shows a range of deviations which include many with only three matches to each of the −10 and −35 hexanucleotide consensus base pairs (15). No sequences indicating σ^{54} dependence are present in the *A. vinelandii glnA* promoter region, which is consistent with genetic evidence that expression of GS activity does not require the *rpoN* (*ntrA*) gene product (43, 52; and A. Toukdarian, unpublished results). Genetic evidence also indicated that the *Eco*RI site adjacent to *glnA* interrupted its expression in *E. coli* (52). This is consistent with the location of the *Eco*RI site within the proposed −10 region of *glnA* (Fig. 4a). This is the first promoter region identified in *A. vinelandii* that does not depend on σ^{54} for expression. Since RNA polymerase purified from *A. vinelandii* has core subunits and a σ^{70} subunit similar to those found in other gram-negative organisms, including enteric bacteria (27), it is expected that many if not most of the *A. vinelandii* promoters would have features similar to those of σ^{70}-dependent promoters of other procaryotes.

Analysis of *glnA* transcripts and β-galactosidase assays of a *glnA-lacZ* fusion strain indicated that expression of *glnA* in *A. vinelandii* is not controlled by levels of fixed nitrogen. This is perhaps not surprising, since ammonium assimilation in this organism is entirely dependent on GS; glutamate dehydrogenase, the alternate pathway for ammonium assimilation in other procaryotes, has not been found (23). The size of the single transcript, approximately 1.5 kb (±20%), correlates with the finding of a probable terminator sequence just downstream of the *glnA* coding region. Therefore, unlike in enteric organisms, expression of the downstream *ntrBC* genes does not occur from the *glnA* promoter.

The inability to isolate glutamine auxotrophs of *A. vinelandii* after introduction of three different types of insertion mutations in the cloned *glnA* gene into the genome suggests that such mutations are lethal. It was possible to detect altered *glnA* regions in strains in which strong selection for the drug resistance encoded by the insert DNA was maintained; however, wild-type DNA was also present, and no growth dependence on added glutamine was observed. Maintenance of both mutated and wild-type alleles is possible because *A. vinelandii* can contain up to 40 or more chromosomes (42). If drug selection was removed, the mutated *glnA* region was lost after several generations of growth. This result is in contrast to the ease of establishing in *A. vinelandii* a number of Tn*5* or Km^r cartridge mutations in several *nif* and *ntr* genes (e.g., references 16 and 52). The proposed lethality of *glnA* mutants is probably not due to polar effects of the insertion on expression of downstream regions because (i) the only transcript detected corresponds in size to the *glnA* region; (ii) the KIXX cartridge, shown to have no effect on expression of downstream genes when cloned in the correct orientation in *A. vinelandii* (G. Selman-Sosa, unpublished results), shows the same unstable maintenance pattern as does Tn*5* inserted into *glnA*; (iii) Tn*5* or KIXX insertions in the *glnA-ntrB* intergenic region (Fig. 1) are stably maintained and gave rise to no discernible phenotype when recombined into the *A. vinelandii* chromosome; and (iv) there are no significant open reading frames with *A. vinelandii* codon usage apparent in the 1 kb of DNA sequence obtained for the region downstream of *glnA* (G. Saunders, unpublished results).

Amino acid auxotrophs of *A. vinelandii* have been difficult to isolate (see reference 24 for a review); only methionine auxotrophs have been reported (9, 22). The Tn*5*-induced methionine auxotrophs that we isolated (22) grew slowly on methionine and were found to be nonviable after a short period of storage (R. Gamal and C. Kennedy, unpublished observations). The two Tn*10*-induced methionine auxotrophs isolated by Contreras and Casadesus (9) reverted to Met^+ at a frequency of 10^{-9}, but their fate upon prolonged storage was not reported. Most Tn*5*-induced *A. vinelandii* mutants that grow on tryptone-enriched medium but not on minimal medium are unable to be characterized as requiring a specific amino acid (22). Thus, it seems possible that the growth requirements of some *A. vinelandii* auxotrophs can be supplied by oligopeptides but not by single amino acids, perhaps because the organism cannot transport certain amino acids very efficiently.

Glutamine uptake assays were performed to test this hypothesis as a possible explanation for our inability to isolate glutamine auxotrophs. When [^{14}C]glutamine was added to cells grown under either N limitation or N excess, an uptake rate of only 0.03 nmol min^{-1} mg of $protein^{-1}$ was measured in 1-min assays; uptake of [^{14}C]glutamate occurred about twice as fast. The rate of glutamine uptake measured in *E. coli* was reported as 2.8 to 54.5 nmol min^{-1}

mg of protein^{-1}, depending on the nitrogen source (55); at minimum, this is equivalent to 170 nmol h^{-1} mg of protein^{-1}. If glutamine is present in *A. vinelandii* at 250 nmol mg (dry weight)$^{-1}$ as is found for *E. coli* (37), then even a rate 10 times that measured here, equivalent to 18 nmol h^{-1} mg of protein^{-1}, would probably not provide sufficient glutamine for good growth of auxotrophs. Such counterselective pressure would ensure maintenance of the wild-type *glnA* gene in the presence of antibiotic selection for the mutated gene. Surprisingly, after several minutes of incubation with [^{14}C]glutamine or [^{14}C]glutamate and under all conditions of N source tested, a significant proportion of the total radioactivity was lost as CO_2, less with glutamine (40%) than with glutamate (90%). This finding could indicate that both amino acids are taken up in *A. vinelandii* but are quickly metabolized, possibly via deamidation-deamination and tricarboxylic acid cycle oxidation. Another possibility is that an extracellular glutaminase converts glutamine to NH_4^+ and glutamate; glutamate is then taken up and oxidized. Further experiments are needed to clarify these possibilities. In any case, the apparent inability of added glutamine to be incorporated into the intracellular amino acid pool may explain why mutations in the *glnA* gene of *A. vinelandii* cannot be stabilized.

Another explanation for the lethality of *glnA* mutations is that GS activity is required for efficient transport of NH_4^+. This view is based on findings that addition of methionine-sulfoximime, an inhibitor of GS activity, prevents efficient transport of the NH_4^+ analog methylammonium into cultures of *A. vinelandii* (2, 19, 35).

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